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The recognition component of the N-end rule pathway

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The N-end rule-based degradation signal, which targets a protein for ubiquitin-dependent proteolysis, comprises a destabilizing amino-terminal residue and a specific internal lysine residue. We report the isolation and functional analysis of a gene (*UBR1*) for the N-end recognizing protein of the yeast *Saccharomyces cerevisiae*. *UBR1* encodes a ~225 kd protein with no significant sequence similarities to other known proteins. Null *ubr1* mutants are viable but are unable to degrade the substrates of the N-end rule pathway. These mutants are partially defective in sporulation and grow slightly more slowly than their wild-type counterparts. The UBR1 protein specifically binds *in vitro* to proteins bearing amino-terminal residues that are destabilizing according to the N-end rule, but does not bind to otherwise identical proteins bearing stabilizing amino-terminal residues.

Key words: degradation signals/N-end rule/protein degradation/ubiquitin/yeast

Introduction

One function of intracellular protein degradation is selective elimination of damaged and otherwise abnormal proteins (Arfin and Bradshaw, 1988; Hershko, 1988; Olson and Dice, 1989). Another is to confer short half-lives on those proteins whose concentrations in the cell must vary with time. Thus, either constitutive or transient metabolic instability is a property of many regulatory proteins (Evans *et al.*, 1983; Banuett *et al.*, 1986; Straus *et al.*, 1987; Murray *et al.*, 1989; Hochstrasser and Varshavsky, 1990). Many other proteins, while long-lived as components of larger macromolecular complexes such as ribosomes and oligomeric proteins, are metabolically unstable in a free, unassociated state (Maicas *et al.*, 1988; Tsay *et al.*, 1988; Kulesh *et al.*, 1989).

At least some proteins are short-lived *in vivo* because they contain sequences (degradation signals) which make these proteins substrates of specific proteolytic pathways. An important component of one degradation signal is the protein's amino-terminal residue (Bachmair *et al.*, 1986). The N-end rule, a code that relates the metabolic stability of a protein to the identity of its amino-terminal residue, is universal in that different versions of it operate in yeast (Bachmair *et al.*, 1986; Bachmair and Varshavsky, 1989), mammals (Reiss *et al.*, 1988; Townsend *et al.*, 1988; Varshavsky *et al.*, 1988; Gonda *et al.*, 1989) and bacteria (J. Tobias and A.Varshavsky, unpublished results).

The N-end rule-based degradation signal in eukaryotes is composed of at least two distinct determinants: the protein's amino-terminal residue and a specific internal lysine residue (Bachmair and Varshavsky, 1989). The second determinant of the signal (a specific lysine residue) is the site of attachment of a multi-ubiquitin chain whose formation on a targeted protein is essential for the protein's subsequent degradation (Chau *et al.*, 1989). The coupling of ubiquitin to other proteins is catalyzed by a family of ubiquitin-conjugating (E2) enzymes, and involves formation of an isopeptide bond between the carboxy-terminal glycine residue of ubiquitin and the ϵ-amino group of a lysine residue in an acceptor protein (Pickart and Rose, 1985; Jentsch *et al.*, 1990). In a multi-ubiquitin chain, ubiquitin itself serves as an acceptor, with several ubiquitin moieties attached sequentially to an initial acceptor protein to form a chain of branched ubiquitin–ubiquitin conjugates (Chau *et al.*, 1989).

Previous work (Bachmair *et al.*, 1986) has predicted the existence of 'N-end-recognizing' factors that select potential proteolytic substrates by binding to their amino-terminal residues. N-end-recognizing proteins have recently been detected in an *in vitro* ubiquitin-dependent proteolytic system derived from mammalian reticulocytes. These proteins have been identified (Reiss *et al.*, 1988; Gonda *et al.*, 1989) as the E3 proteins that were previously shown to bind proteolytic substrates prior to their ubiquitination by a subset of ubiquitin-conjugating (E2) enzymes (reviewed by Finley *et al.*, 1988; Hershko, 1988; Ciechanover and Schwartz, 1989).

We report the first cloning and functional dissection of a gene encoding an N-end-recognizing (E3) protein. The *UBR1* gene of the yeast *Saccharomyces cerevisiae* encodes a 225 kd protein that has no significant sequence similarity to other known proteins. *In vivo* and *in vitro* analyses identify UBR1 as the recognition component of the N-end rule pathway.

Results

Isolation of the ubr1 mutant

In eukaryotes, ubiquitin–X–β-galactosidase (Ub-X-βgal) fusion proteins are precisely deubiquitinated either *in vivo* or in cell-free extracts by an endogenous processing protease to yield X-βgal test proteins bearing the residue X at the amino terminus (Bachmair *et al.*, 1986; Gonda *et al.*, 1989). In contrast to the function of ubiquitin at later stages of the degradative pathway, the role of ubiquitin in these engineered ubiquitin fusions is simply to allow the generation of X-βgals or other sets of otherwise identical proteins bearing different amino-terminal residues. Depending on the nature of X, the X-βgal proteins are either long-lived or metabolically unstable, with destabilizing amino-terminal residues

conferring short half-lives on the corresponding X-βgals (Bachmair *et al.*, 1986; Bachmair and Varshavsky, 1989; Gonda *et al.*, 1989). This amino-terminal degradation signal is manifested as the N-end rule.

The *S.cerevisiae* strain BWG9a-1 (Table I) carrying a 2μ-based plasmid that expressed Ub-Arg-βgal (Bachmair *et al.*, 1986) was used to screen for mutants defective in the N-end rule pathway. Since Arg-βgal (produced by the *in vivo* deubiquitination of Ub-Arg-βgal) is normally short-lived *in vivo* ($t_{1/2}$ of ~2 min at 30°C; Bachmair *et al.*, 1986), its steady-state level in wild-type cells is low, and the corresponding yeast colonies are white on plates containing the chromogenic βgal substrate X-Gal. In constrast, cells expressing long-lived X-βgals such as Met-βgal ($t_{1/2}$ of ~20 h; produced from Ub-Met-βgal; Bachmair *et al.*, 1986) have high βgal activity and form blue colonies on X-Gal plates. Cells expressing Arg-βgal were mutagenized with ethyl methanesulfonate (EMS) and plated on X-Gal plates (see Materials and methods). We screened for blue colonies, among which we expected to find mutants with defects in the degradation of Arg-βgal. Putative mutants identified on X-Gal plates were retested by measuring βgal activity in crude extracts from these cells (see Materials and methods). The mutants were also tested for the stabilization of other X-βgals by removing the Ub-Arg-βgal expressing plasmid and transforming the mutants with plasmids expressing other Ub-X-βgal proteins. One of the mutants appeared to stabilize all of the normally short-lived X-βgal proteins. However, the inefficiently deubiquitinated, short-lived Ub-Pro-βgal (Bachmair *et al.*, 1986), whose metabolic instability is independent of the N-end rule-based degradation signal (E.Johnson and A.Varshavsky, unpublished results), remained short-lived in the mutant. We confirmed that the increased βgal activity in this mutant was indeed due to metabolic stabilization of the normally short-lived X-βgals by determining the *in vivo* half-lives of several X-βgal proteins in the mutant, using pulse–chase analysis (data not shown; see also below). This mutant, named *ubr1*, had the properties expected of a substrate recognition defect in the N-end rule pathway, and was therefore chosen for further study.

The UBR1 gene

The *ubr1* mutation was recessive (data not shown), allowing the wild-type *UBR1* gene to be cloned by complementation. *ubr1* cells expressing Arg-βgal (which form blue colonies on X-Gal plates; see above) were transformed with a yeast genomic DNA library and plated on X-Gal plates to identify phenotypically wild-type (low levels of βgal) colonies, which are white on these plates (see Materials and methods). Plasmid DNA isolated from two such colonies contained identical yeast DNA inserts. A portion of the insert DNA was able to target a marker gene (*LEU2*) to the chromosomal locus of the original *ubr1* mutation (see Materials and methods), indicating that the cloned gene either encompassed or was closely linked to the site of the original mutation.

Several fragments of the cloned insert did not complement the *ubr1* mutation (see Materials and methods). Therefore, the entire 8.65 kb insert was sequenced, revealing an open reading frame of 5.85 kb that encoded a 1950 residue protein with a calculated molecular mass of 225 kd (Figure 1). The position of the start ATG codon was inferred so as to yield the longest open reading frame. No ATGs occur in any of the three forward reading frames upstream of this putative initiator ATG until position −482 (Figure 1). A single ~7 kb transcript was detected by Northern hybridization analysis of total yeast RNA probed with the ~4 kb *Nsi*I DNA fragment (Figure 2A) that included the 3′ two-thirds of the *UBR1* coding region (data not shown). This *UBR1* RNA was present at similar levels in *MAT***a** and *MATα* haploids and *MAT***a**/*MATα* diploids, and was not significantly induced either by heat stress or in stationary phase (data not shown). The *UBR1* gene has a relatively low bias for codons that are preferred in highly expressed yeast genes. The Codon Adaptation Index for *UBR1* is 0.146 (calculated according to Sharp and Li, 1987), suggesting a low level

Table I. Yeast strains

Strain	*Genotype*
BWG9a-1	*MATa his4 ura3 ade6*
BWG1-7a	*MAT***a** *his4 ura3 ade1 leu2*
IW100	*MATa ubr1 his4 ura3 ade6*
IW104	*MAT***a** *ubr1 his4 ura3 ade6 leu2*
DF5[a]	*MATa/MATa trp1-1/trp1-1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 gal/gal*
BBY40[b]	*MATa/MATa ubr1-Δ1::LEU2/UBR1 trp1-1/trp1-1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys 2-801/lys2-801 gal/gal*
BBY41[b]	*MATa/MATa ubr1-Δ1::LEU2/UBR1 trp1-1/trp1-1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 gal/gal*
BBY45[c]	*MAT***a** *trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*
BBY46[c]	*MATa ubr1-Δ1::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*
BBY47[c]	*MAT***a** *ubr1-Δ1::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*
BBY48[c]	*MATa trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*
BBY49[d]	*MAT***a** *ubr1-Δ1::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*
BBY53[e]	*MATa/MATa ubr1-Δ1::LEU2/ubr1-Δ1::LEU2 trp1-1/trp1-1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 gal/gal*
BBY58[f]	*MATa/MATa ubr1-Δ1::LEU2/ubr1-Δ1::LEU2 trp1-1/trp1-1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 gal/gal*

[a]Finley *et al.* (1987)
[b]Independent isolates produced by transforming DF5 with a 5 kb *Hin*dIII fragment from the plasmid pSOB30 and selecting Leu$^+$ transformants.
[c]Derived from diploid strain BBY40 as products of a single meiotic tetrad.
[d]A segregant derived from sporulation of BBY41.
[e]Produced by mating BBY46 and BBY47 and picking zygotes with a micromanipulator.
[f]Produced by mating BBY46 and BBY49 and picking zygotes with a micromanipulator.

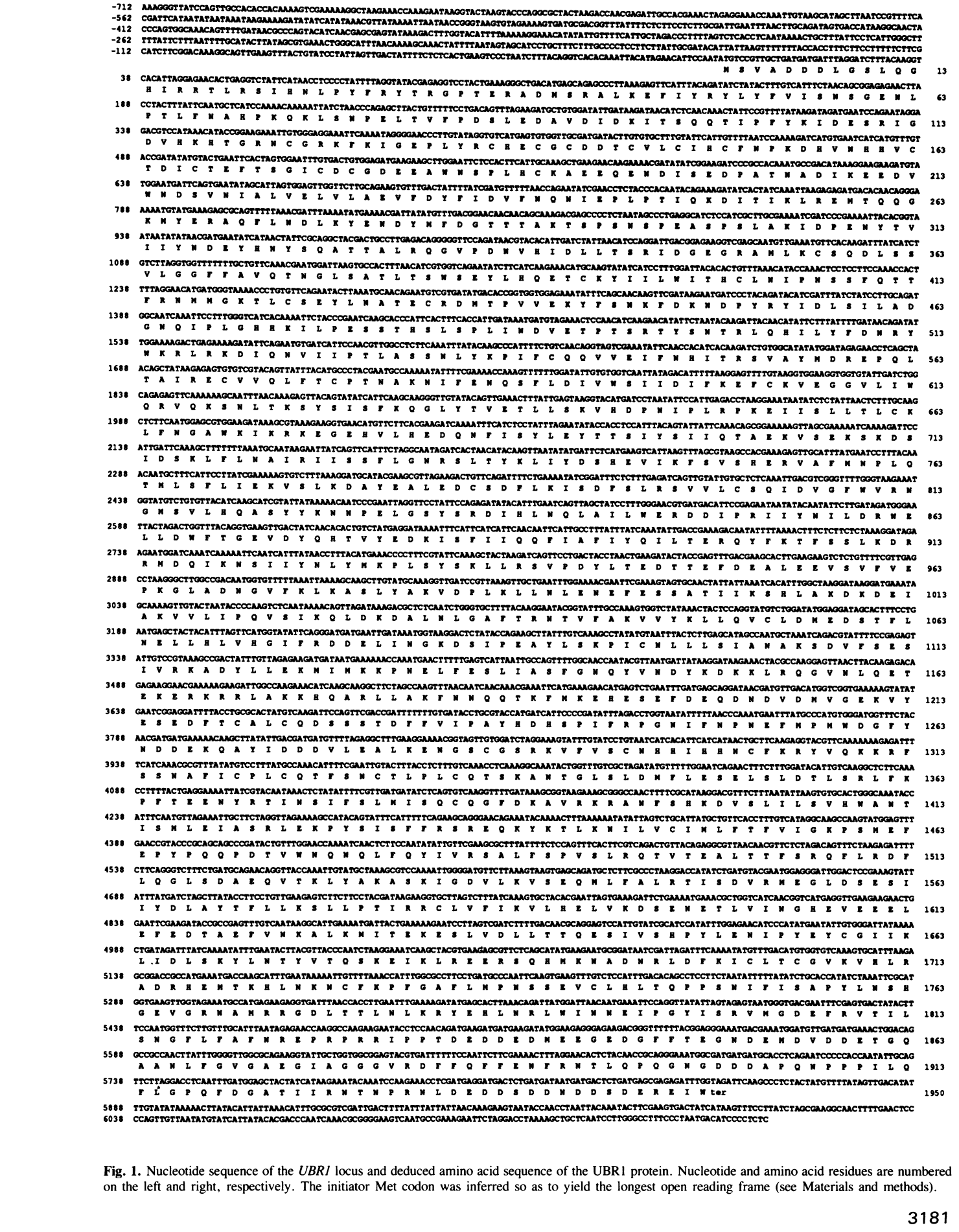

Fig. 1. Nucleotide sequence of the *UBR1* locus and deduced amino acid sequence of the UBR1 protein. Nucleotide and amino acid residues are numbered on the left and right, respectively. The initiator Met codon was inferred so as to yield the longest open reading frame (see Materials and methods).

of *UBR1* expression in wild-type cells. When the *UBR1* gene was expressed from a high copy (2μ-based) plasmid (pSOB33 or pSOB35; see Materials and methods) in a *ubr1-Δ1* strain (see below), a protein of ~200 kd was detected by Coomassie staining of SDS–PAGE fractionated proteins from whole cell extracts. The presence of the ~200 kd protein depended on the presence of the *UBR1* insert in the plasmid, confirming that the cloned DNA fragment directs the synthesis of an appropriately sized protein. [In these gels, UBR1 migrates slightly faster than [^{14}C]methylated myosin (Amersham) but comigrates with prestained myosin (Diversified Biotech); see also Figure 6A].

Computer-aided comparison of the *UBR1* nucleotide sequence (and the predicted amino acid sequence) to sequences in databases (see Materials and methods) revealed no significant similarities to sequenced genes or gene products. Furthermore, no *UBR1* cross-hybridizing sequences (other than *UBR1* itself) were detected in the *S.cerevisiae* genome by low-stringency Southern hybridization (data not shown). The UBR1 protein does contain several sequence motifs characteristic of other known proteins, including a stretch encoding $(LeuX_6)_4$ (a putative leucine zipper) (Landschulz *et al.*, 1988) at residues 775–796, and a cluster of six cysteines at residues 136–151, spaced identically to the cysteines of the HIV tat protein, which apparently play a role in tat dimerization (Frankel *et al.*, 1988). In the absence of additional evidence, the functional significance of these sequences in UBR1 is unknown, in part because the probability of random occurrence of such motifs in a large (1950 residues) protein is relatively high.

Construction of a ubr1 null mutant

A *ubr1* null allele was constructed *in vitro* by replacing ~5 kb of the *UBR1* coding region with a ~2.2 kb fragment carrying the *LEU2* gene (Figure 2A and Materials and methods). A linear DNA fragment that included the disruption (which lacked >85% of the *UBR1* coding region) was used to transform DF5, a *leu2/leu2 UBR1/UBR1* diploid strain (Table I). Leu$^+$ transformants, expected to be heterozygous at the *UBR1* locus, were sporulated; tetrad dissection yielded four viable spores whose Leu$^+$ phenotype segregated 2:2, indicating that the *UBR1* gene is not essential for either spore germination or vegetative growth. Southern hybridization analysis of a transformant and its segregants confirmed the predicted structure of the *ubr1-Δ1* allele by showing the presence of restriction endonuclease sites diagnostic of the transplacement (Figure 2B), and Northern analysis confirmed that the *UBR1* message was not present in the deletion strain (data not shown).

Phenotypic analysis of the ubr1-Δ1 mutant

Plasmids expressing various Ub-X-βgal proteins were transformed into congenic *UBR1* and *ubr1-Δ1* strains. Met-βgal and other X-βgals bearing stabilizing amino-terminal residues remained long-lived in the *ubr1-Δ1* strain (data not shown). As expected from the phenotype of the original *ubr1* mutant, all of the normally short-lived X-βgals were metabolically stabilized in the *ubr1-Δ1* strain (Figure 3A and B and data not shown), while the inefficiently deubiquitinated, short-lived Ub-Pro-βgal was degraded in this mutant with wild-type kinetics (Figure 3C). Moreover, the pattern of Ub-Pro-βgal multi-ubiquitination (before degradation) in the

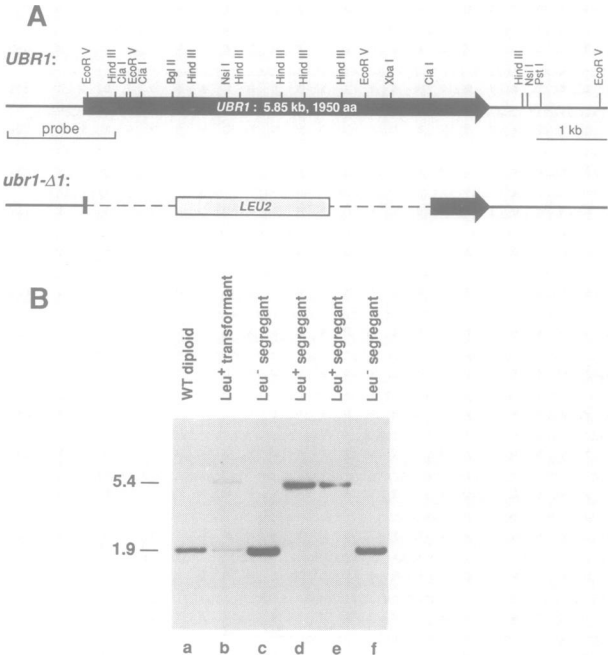

Fig. 2. Deletion of the *UBR1* gene. (**A**) Restriction map of the 8.65 kb genomic DNA fragment encompassing the wild-type *UBR1* gene, and the structure of the *ubr1-Δ1* allele (see Materials and methods). (**B**) Southern hybridization analysis of the *ubr1-Δ1* (*ubr-Δ1::LEU2*) allele. Genomic DNA from the parental diploid strain (DF5) homozygous at the *UBR1* locus (**lane a**), from a Leu$^+$ transformant (BBY40) of DF5 (**lane b**), and from four haploid products (BBY45–BBY48) (**lanes c–f**) of a single meiotic tetrad of BBY40 was digested with *Hin*dIII and analyzed by Southern hybridization using a ~1.5 kb probe extending upstream from the *Hin*dIII site at position +542 (see A). Shown on the left are the positions of *Hin*dIII fragments from the *UBR1* (1.9 kb) and *ubr1-Δ1* (5.4 kb) alleles that overlap the hybridization probe. See Table I for the complete genotypes of the strains.

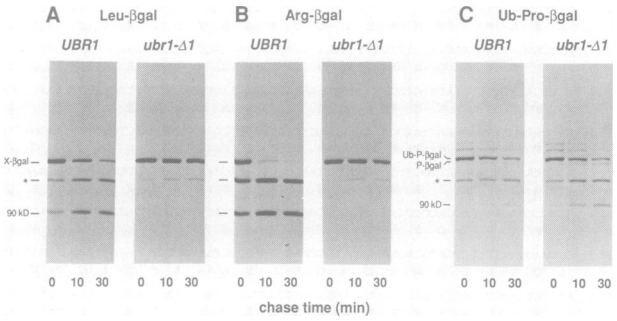

Fig. 3. Comparison of metabolic stabilities of X-βgal and Ub-Pro-βgal proteins in *UBR1* and *ubr1-Δ1* strains. (**A**) Exponential cultures of either BBY45 (*UBR1*) or BBY47 (*ubr1-Δ1*) *S.cerevisiae* strains carrying a plasmid which expresses Ub-Leu-βgal, were labeled with [^{35}S]methionine for 5 min at 30°C followed by a chase in the presence of cycloheximide and excess cold methionine for 0, 10 or 30 min. Extracts were immunoprecipitated with a monoclonal antibody to βgal, and analyzed by SDS–PAGE (see Materials and methods). (**B**) Same as in A but with a plasmid expressing Ub-Arg-βgal. (**C**) Same as in A but with a plasmid expressing Ub-Pro-βgal. An asterisk denotes an unknown *S.cerevisiae* protein that crossreacts with the monoclonal antibody to βgal (Bachmair *et al.*, 1986). '90 kd' indicates a long-lived βgal cleavage product that is specific for short-lived X-βgals and represents an alternative metabolic fate for short-lived X-βgals in yeast (Bachmair *et al.*, 1986; Chau *et al.*, 1989). In A and B, the fluorograms from the *UBR1* strain were exposed 2- to 3-fold longer than those from the (*ubr1-Δ1*) mutant.

ubr1-Δ1 mutant was indistinguishable from that in wild-type cells (Figure 3C). The recognition of Ub-Pro-βgal as a proteolytic substrate is independent of the N-end rule-based degradation signal, and has recently been shown to be mediated by the slowly removed ubiquitin moiety of Ub-Pro-βgal (E.Johnson and A.Varshavsky, unpublished results). Taken together, these data indicated that the *ubr1-Δ1* mutant was affected in an early substrate recognition step of the N-end rule pathway but apparently not in the later steps of multi-ubiquitination or degradation of the substrate. This was consistent with the possibility, to be confirmed below, that the *UBR1* gene encodes the previously postulated N-end-recognizing protein (Bachmair *et al.*, 1986) that distinguishes between potential proteolytic substrates bearing destabilizing or stabilizing amino-terminal residues.

Detailed phenotypic characterization of the *ubr1-Δ1* mutant uncovered no significant differences from wild-type cells in sensitivity to chronic or acute heat stress; in survival during stationary phase or nitrogen or carbon starvation; in ability to utilize various carbon sources or grow anaerobically; in survival of freeze–thaw treatments or exposures to cytotoxic concentrations of ethanol; or in sensitivity to chronic exposure to canavanine, an arginine analog (data not shown; see Materials and methods). Furthermore, while the *ubr1-Δ1* mutant was unable to degrade the normally short-lived, engineered substrates of the N-end rule pathway such as X-βgals (see above), no defect in bulk protein degradation, measured by following the release of acid soluble ^{35}S from pulse-labeled normal or abnormal (canavanine-containing) proteins, could be demonstrated (data not shown; see Materials and methods). Moreover, none of the naturally short-lived yeast proteins that were identified by pulse–chase and O'Farrell two-dimensional electrophoresis was metabolically stabilized in the *ubr1-Δ1* mutant (data not shown).

One phenotypic characteristic of the *ubr1-Δ1* mutant was a slightly increased doubling time. The difference was too small to be seen from a comparison of individual growth curves over a small number of generations, but was detected by following the growth of a mixed culture of a *ubr1-Δ1* (*LEU2*) and a congenic *UBR1* (*leu2*) strain. The two strains were grown in the same flask in rich (YPD) medium, and kept in exponential phase by daily dilution into fresh medium. The ratio of *ubr1-Δ1* to *UBR1* cells in the mixed population was monitored by plating diluted samples of the culture on SD medium either containing or lacking leucine. The relative content of *ubr1-Δ1* (*LEU2*) cells in the mixed population was determined as the ratio of the number of Leu^+ colonies to the total number of colonies recovered from the culture. The results of one such experiment are shown in Figure 4A. (Three repetitions of this competition experiment yielded the same result.) The proportion of *ubr1-Δ1* cells gradually decreased in the growing population, reflecting a slightly (~2%) longer doubling time of the *ubr1-Δ1* cells relative to that of their congenic *UBR1* counterparts. Similar results were also obtained with a different haploid *ubr-Δ1* strain (BBY46; data not shown).

In addition, we constructed a *ubr1-Δ1/ubr1-Δ1* diploid strain (BBY58, see Table I) from independently derived *ubr1-Δ1* haploids. This diploid strain also showed an increased doubling time in a co-cultivation experiment with the parental *UBR1/UBR1* diploid (Figure 4B), indicating that the effect was not due to an unrelated (non-*ubr1*) recessive mutation (which would be expected to be heterozygous, and therefore phenotypically normal, in the *ubr1-Δ1/ubr1-Δ1* diploid). Moreover, no difference in doubling time was observed when the heterozygous (*ubr1-Δ1/UBR1*) diploids BBY40 or BBY41 (Table I) were co-cultivated with the homozygous (*UBR1/UBR1*) parental diploid DF5 (data not shown). This confirmed that the strains from which the *ubr1-Δ1* haploids and homozygous (*ubr1-Δ1/ubr1-Δ1*) diploids were derived carried no mutations with dominant detrimental effects on growth, and also that the presence of the *LEU2* gene in the *ubr1-Δ1* deletion/disruption was not the source of the growth defect. In addition, a haploid strain carrying a different gene disrupted by the same *LEU2* allele (*ubi4-Δ2::LEU2*; Finley *et al.*, 1987) in the same (*UBR1*) genetic background showed no growth disadvantage in a similar co-cultivation experiment with the congenic *UBR1* (*leu2*) strain (data not shown).

Another abnormal phenotypic characteristic of the *ubr1-Δ1* mutant was a defect in sporulation of *ubr1-Δ1/ubr1-Δ1* diploids. Specifically, although wild-type numbers of asci were produced by the mutant, the proportion of aberrant (two-spored) asci was much higher in the *ubr1-Δ1/ubr1-Δ1* than in the congenic *UBR1/UBR1* strain (Table II). At the same time, the *ubr1-Δ1* spores from either normal or aberrant asci were apparently normal meiotic products, and germinated at the same frequency (~98%) as those from the wild-type (*UBR1*) strain.

UBR1 binds to substrates of the N-end rule pathway

To test whether UBR1 specifically interacts with substrates of the N-end rule pathway, two assays were used. In the first, we asked whether UBR1 could be specifically crosslinked to dihydrofolate reductase (X-DHFR) derivatives bearing either a destabilizing (Leu) or a stabilizing (Met) amino-terminal residue. As observed with X-βgals, the *in vivo* degradation of Leu-DHFR is dependent on the presence of a wild-type *UBR1* allele (R.Baker and A.Varshavsky, unpublished data). X-DHFR rather than X-βgal proteins were used in these experiments, since the smaller size of

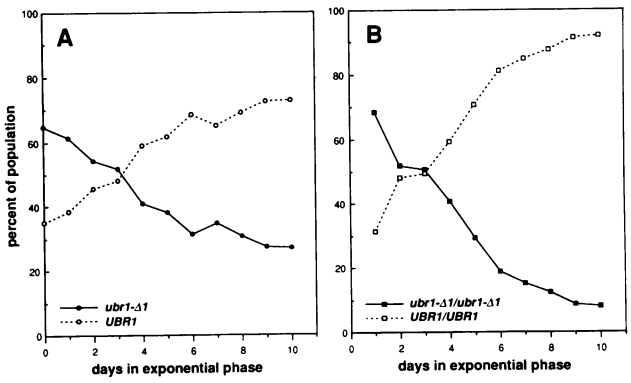

Fig. 4. Growth advantage of *UBR1* over *ubr1-Δ1* strains in mixed cultures. (**A**) Exponential cultures of BBY45 (*UBR1*, open circles) and BBY47 (*ubr1-Δ1::LEU2*, closed circles) strains were inoculated into a 500 ml flask containing 100 ml of rich medium (YPD), and grown with shaking at 30°C. The culture was diluted daily into a 500 ml flask containing fresh medium so that A_{600} of the culture never exceeded 1.5. Samples of the culture were taken daily, diluted appropriately, and spread on minimal (SD) medium plates either lacking or containing leucine. Plates were incubated at 30°C to allow colony formation and scoring of Leu^+ (*ubr1-Δ1*) and Leu^- (*UBR1*) viable cells in the mixed culture. (**B**) Same as in A but with the congenic diploid strains DF5 (*UBR1/UBR1*, open squares) and BBY58 (*ubr1-Δ1:: LEU2/ubr1-Δ1::LEU2*, closed squares).

Table II. Abnormal sporulation of the *ubr1-Δ1/ubr1-Δ1* diploid

Relevant genotype	Percent of cells sporulated (mean ± s.d.)	Percent of asci with two spores (mean ± s.d.)
UBR1/UBR1	17 ± 2	5 ± 4
ubr1-Δ1/ubr1-Δ1	23 ± 1	38 ± 3

DF5 (*UBR1/UBR1*) and BBY53 (*ubr1-Δ1/ubr1-Δ1*) strains were sporulated for 3 days at 30°C in liquid media (2% potassium acetate). The proportion of 2-spored asci and efficiency of sporulation were determined using a hemacytometer. Shown are the mean value and standard deviation (s.d.) from three measurements of the same culture. Both 2- and 4-spored asci gave rise to haploid, ~98% viable spores.

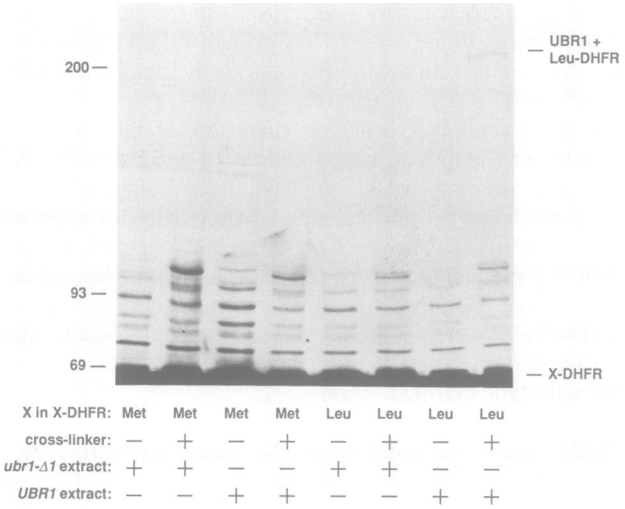

Fig. 5. Crosslinking of the UBR1 protein to a substrate of the N-end rule pathway. [^{35}S]methionine-labeled Ub-Met-DHFR and Ub-Leu-DHFR proteins were purified from *E.coli* and deubiquitinated by incubation in an extract prepared from a *ubr1-Δ1* yeast strain (see Materials and methods). The resultant Met-DHFR and Leu-DHFR proteins, which differed exclusively at their amino termini (Bachmair and Varshavsky, 1989), were incubated for 15 min at 0°C with extracts from either *ubr1-Δ1* cells or cells overexpressing the UBR1 protein. These incubations were carried out either in the presence or in the absence of the crosslinker *bis* (sulfosuccinimidyl) suberate. Crosslinking was quenched with ethanolamine, and the samples were analyzed by SDS−PAGE and fluorography (see Materials and methods). Protein bands present in all lanes above the (overexposed) DHFR band are contaminating *E.coli* proteins. Molecular masses (in kd) of ^{14}C-labeled protein markers (Amersham) are shown on the left.

DHFR was expected to simplify electrophoretic fractionation of crosslinked UBR1−substrate complexes. A similar crosslinking assay has been used to address the recognition properties of a ubiquitin-dependent proteolytic system in mammalian reticulocytes (Hershko *et al.*, 1986; Reiss *et al.*, 1988).

^{35}S-labeled Leu-DHFR and Met-DHFR were prepared by purifying the corresponding Ub-X-DHFR proteins (Bachmair and Varshavsky, 1989) from *Escherichia coli* labeled with [^{35}S]methionine, and deubiquitinating them *in vitro* with an extract from a *ubr1-Δ1* yeast strain (see Materials and methods). Extracts from either a *ubr1-Δ1* strain or a UBR1 overproducing strain (carrying *UBR1* on a high copy plasmid) were added to the ^{35}S-labeled Leu-DHFR or Met-DHFR, together with the crosslinking reagent *bis* (sulfosuccinimidyl) suberate. ^{35}S-labeled products were analyzed by SDS−PAGE and fluorography (Figure 5). A discrete ^{35}S-labeled species that migrated more slowly than uncrosslinked UBR1 was formed under the above conditions only if the crosslinker, UBR1, and [^{35}S]Leu-DHFR were present (Figure 5). The simplest interpretation of this result is that the unlabeled 225 kd UBR1 protein, when present in the extract, binds to the ~22 kd [^{35}S]Leu-DHFR but not to the otherwise identical [^{35}S]Met-DHFR.

To verify and extend this result, we used a second assay involving co-immunoprecipitation. The epitope tagging method (Munro and Pelham, 1984; Field *et al.*, 1988; Finley *et al.*, 1989) was employed to allow immunoprecipitation of the UBR1 protein. The *UBR1* gene was mutated *in vitro* so that it encoded UBR1 extended at its carboxyl terminus by nine residues [derived from hemagglutinin (ha) of influenza virus] that are recognized by the monoclonal antibody 12CA5 (Field *et al.*, 1988; see also Materials and methods). The modified *UBR1* gene (*UBR1−ha*), when expressed from a plasmid, complemented the X-βgal degradation defect of the *ubr1-Δ1* mutant to the same extent as the unmodified *UBR1* gene expressed from an otherwise identical plasmid (data not shown), indicating that the ha tag did not significantly interfere with UBR1 function. The UBR1−ha protein, when expressed from a high copy plasmid, was selectively immunoprecipitated from cell extracts with the anti-ha monoclonal antibody (Figure 6A).

In the co-immunoprecipitation experiment (Figure 6B), extracts from cells overexpressing either UBR1-ha or untagged UBR1 were mixed with [^{35}S]X-βgal bearing either a destabilizing (Arg) or a stabilizing (Val) amino-terminal residue, followed by immunoprecipitation with the anti-ha antibody. Arg-βgal, but not Val-βgal, was precipitated, but only if the tagged UBR1−ha protein was present during incubation (Figure 6B). We conclude that UBR1, which is essential for the *in vivo* degradation of substrates of the N-end rule pathway, specifically binds to these substrates *in vitro*.

UBR1 is a rate-limiting component of the N-end rule pathway

In the course of using yeast strains overproducing UBR1 for the above *in vitro* analyses, we observed that cells expressing a short-lived X-βgal protein and overexpressing UBR1 had significantly lower levels of βgal than congenic cells with a wild-type content of UBR1 (Figure 7A). This result suggested that UBR1 is normally rate-limiting for X-βgal degradation *in vivo*. Pulse−chase analysis confirmed that the half-life of the metabolically unstable Tyr-βgal (Bachmair *et al.*, 1986) was shortened in cells overexpressing UBR1 (Figure 7B). In addition, the relative level of multiply ubiquitinated derivatives of Tyr-βgal (its putative degradation intermediates; Chau *et al.*, 1989; Gonda *et al.*, 1989) was increased in UBR1 overexpressing cells (Figure 7B). Thus, under these conditions the rate of degradation of multi-ubiquitinated substrates of the N-end rule pathway

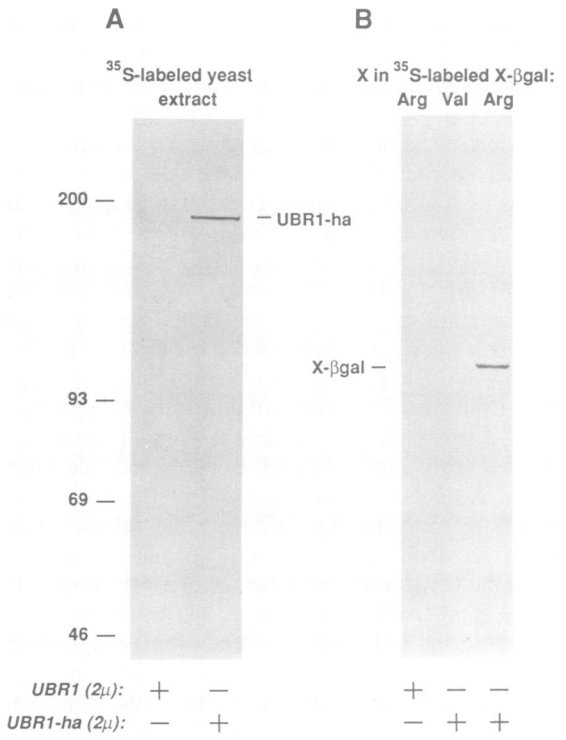

Fig. 6. Using an epitope tagged UBR1−ha protein to detect UBR1−substrate binding. (**A**) Immunoprecipitation of UBR1−ha (UBR1 tagged with ha, a nine residue peptide derived from hemagglutinin of influenza virus). Exponential culture of BBY47 (*ubr1-Δ1*) transformed with 2μ-based plasmids carrying either *UBR1* (pSOB35) or *UBR1−ha* (pSOB37) were labeled with [^{35}S]methionine for 15 min at 30°C, followed by extraction, immunoprecipitation with an anti-ha monoclonal antibody, and electrophoretic analysis (see Materials and methods). Molecular masses (in kd) of ^{14}C-labeled protein markers (Amersham) are shown on the left. (**B**) Specific co-immunoprecipitation of UBR1−ha and a substrate protein. [^{35}S]methionine-labeled Ub-Arg-βgal and Ub-Val-βgal were purified from *E.coli* and deubiquitinated by incubation in an extract prepared from the BBY47 (*ubr1-Δ1*) yeast strain. The resultant Arg-βgal and Val-βgal proteins were incubated for 1 h at 0°C with extracts from *ubr1-Δ1* (BBY47) cells transformed with high-copy plasmids expressing either UBR1 or UBR1−ha, followed by immunoprecipitation with an anti-ha monoclonal antibody and electrophoretic analysis (see Materials and methods). Overexposures of these fluorograms showed Val-βgal was not immunoprecipitated from the (UBR1−ha)-containing extract over background levels (the amount of X-βgal immunoprecipitated with the anti-ha antibody from the extract containing untagged UBR1).

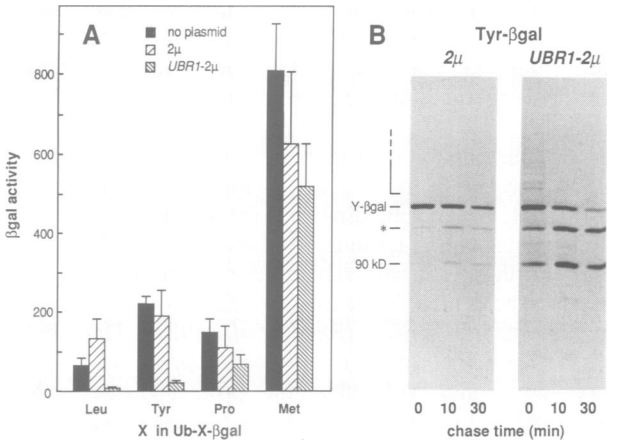

Fig. 7. Effect of overproduction of UBR1 on metabolic instability of X-βgal proteins. (**A**) βgal activity in yeast strains overexpressing UBR1. BBY45 (*UBR1*)-based strains carrying plasmids expressing different Ub-X-βgal proteins were transformed with either a control high copy (2μ-based) vector (YEplac112) or an otherwise identical vector expressing UBR1 (pSOB35). Extracts prepared from exponentially growing cells were assayed for βgal activity using ONPG (see Materials and methods). Values shown are the means of at least three independent measurements. Standard deviations are shown above each bar. (**B**) Enhanced metabolic instability of Tyr-βgal in yeast overexpressing UBR1. Exponential cultures of the BBY45 (*UBR1*) strain carrying plasmids expressing Ub-Tyr-βgal and either a control 2μ-based vector or an otherwsie identical vector encoding UBR1 (pSOB35) were labeled with [^{35}S]methionine for 5 min at 30°C, followed by a chase in the presence of 0.5 mg/ml cycloheximide for 0, 10 or 30 min, extraction, immunoprecipitation with a monoclonal antibody to βgal and electrophoretic analysis (see Materials and methods).

begins to limit the overall rate of substrate flux through the pathway.

Discussion

The UBR1 protein

We have isolated a yeast mutant that stabilizes normally short-lived substrates of the N-end rule pathway such as Arg-βgal. The wild-type version of the gene (*UBR1*) that is defective in the mutant has been cloned and shown to encode a 225 kd protein that has no homologs in current databases. The UBR1 protein binds *in vitro* to substrates of the N-end rule pathway, with the specificity of binding determined at least in part by the nature of the substrate's amino-terminal residue. These results identify UBR1 as the recognition component of the N-end rule pathway.

Proteins with the properties of UBR1 were predicted by Bachmair *et al.* (1986), and were first detected by Reiss *et al.* (1988) and Gonda *et al.* (1989) in an *in vitro* ubiquitin-dependent proteolytic system derived from rabbit reticulocytes. These proteins were identified as the E3 proteins that had previously been shown to be required for ubiquitination of proteolytic substrates by specific ubiquitin-conjugating (E2) enzymes and to contain the substrate-binding site of the proteolytic pathway (Hershko *et al.*, 1983, 1986).

Three distinct types of E3 activity have been detected in the above *in vitro* system using assays based on selective inhibition of the degradation of specific proteins by dipeptides bearing different destabilizing amino-terminal residues. The type I E3 activity is specific for the positively charged destabilizing amino-terminal residues Arg, Lys and His (Reiss *et al.*, 1988; Gonda *et al.*, 1989). The type II activity is specific for the bulky hydrophobic destabilizing amino-terminal residues Phe, Trp, Tyr and Leu (and Ile in yeast) (Reiss *et al.*, 1988; Gonda *et al.*, 1989; R.Baker and A.Varshavsky, unpublished results). The type III activity is specific for the amino-terminal residues Ala, Ser and Thr, which share the properties of small size and lack of charge (Gonda *et al.*, 1989). The Ala, Ser and Thr residues are destabilizing in reticulocytes but stabilizing in yeast, implying that *S.cerevisiae* lacks type III activity (Bachmair *et al.*, 1986; Bachmair and Vashavsky, 1989; Gonda *et al.*, 1989).

The *ubr1-Δ1* mutant metabolically stabilizes X-βgals bearing either type I or type II destabilizing amino-terminal residues. Furthermore, we have demonstrated that the UBR1 protein binds *in vitro* to representatives of both type I and type II substrates (see Results). Since the type I and type II binding activities can be independently inhibited in both

yeast UBR1 (R.Baker and A.Varshavsky, unpublished results) and in a mammalian counterpart of UBR1 (Reiss *et al.*, 1988; Gonda *et al.*, 1989), it is likely that the corresponding binding sites are distinct in both of these proteins.

Partial purification of a rabbit E3 has shown it to be a ~180 kd protein (Hershko *et al.*, 1986) that, like the 225 kd yeast UBR1, contains binding sites for both type I and type II destabilizing amino-terminal residues (Reiss and Hershko, 1990). Furthermore, the rabbit E3 protein (whose gene remains to be cloned) binds to a specific ubiquitin-conjugating (E2) enzyme *in vitro* (Reiss *et al.*, 1989). Thus, both the yeast and the rabbit E3 proteins can be viewed as 'recognition' subunits of a ubiquitin–protein ligase complex whose catalytic component is a specific ubiquitin-conjugating (E2) enzyme. In a model for substrate recognition by the N-end rule pathway (Bachmair and Varshavsky, 1989) the recognition component of the pathway (either an E3 protein alone or in a complex with an E2 enzyme) has a binding site for the substrate's destabilizing amino-terminal residue, and a lysine binding site. The latter site binds the second-determinant lysine residue of the N-end rule-based degradation signal (see Introduction). Occupation of both of these sites by a proteolytic substrate is postulated to be required for multi-ubiquitination to commence at the bound lysine of the substrate (Bachmair and Varshavsky, 1989). It remains to be determined whether E3 proteins such as UBR1 contain a lysine binding site, in addition to the type I and type II binding sites for destabilizing amino-terminal residues (and presumably also an E2 enzyme binding site), or whether the lysine binding site is located in the E3-bound E2 enzyme.

The relative ease of overexpression of the UBR1 protein in yeast, and the availability of an epitope tagged UBR1 (see Results) will allow purification of the tagged UBR1–ha protein by affinity chromatography using the anti-ha monoclonal antibody. Having significant amounts of the UBR1–ha protein should allow detailed functional mapping of UBR1 and identification, via direct binding assays, of UBR1-interacting yeast proteins, including the relevant E2 enzyme(s).

A note on terminology: we suggest the name 'N-recognin' for proteins that are functionally equivalent to the N-end-recognizing yeast UBR1 protein. More generally, the term 'recognin' is proposed to denote recognition components of intracellular proteolytic pathways (either independent of or dependent on ubiquitin), with specific prefixes (e.g. N-recognin) distinguishing various recognins. Recent evidence (reviewed by Hershko, 1988) suggests that the class of recognins thus defined contains more than one functionally distinct member. Moreover, certain previously characterized proteins, in particular some of the homologs of heat shock proteins (Chiang *et al.*, 1989), are likely to function as specific recognins.

On the function of the N-end rule pathway

The N-end rule pathway is inactive in the *ubr1-Δ1* mutant (see Results). The viability of this mutant, and its wild-type sensitivity to a variety of metabolic and physical stresses (see Results) indicate that this pathway is not essential in growing yeast cells or germinating spores.

However, the lack of the N-end rule pathway does have a subtle effect on sporulation, inasmuch as the proportion of aberrant (two-spore) asci was much higher in a *ubr1-Δ1/ubr1-Δ1* strain than in a congenic *UBR1/UBR1* strain (Table II). It is possible that this sporulation defect is due to metabolic stabilization, in the *ubr1-Δ1* mutant, of a protein or proteins whose rapid degradation is required for efficient execution of a step in spore formation.

Another characteristic of the phenotype of the *ubr1-Δ1* mutant is its slightly decreased growth rate (Figure 4). Both the sporulation and the growth rate defects of the *ubr1-Δ1* mutant, while far from dramatic, would have been sufficient to retain *UBR1*, and by inference the N-end rule pathway, in the course of evolution. At the same time, these subtle defects have not illuminated specific functions of the N-end rule pathway. The problem is exacerbated by the lack of known physiological substrates of this pathway. Specifically, the *ubr1-Δ1* mutant, while unable to degrade the normally short-lived engineered substrates of the N-end rule pathway such as X-βgals, showed no defect in bulk protein degradation, measured by following the release of acid-soluble ^{35}S from pulse-labeled normal or abnormal (canavanine-containing) proteins. Moreover, none of the naturally short-lived yeast proteins that were identified by pulse–chase and O'Farrell two-dimensional electrophoresis was metabolically stabilized in the *ubr1-Δ1* mutant (data not shown). Since the degradation of abnormal (canavanine-containing) proteins is known to be ubiquitin-dependent, at least in part, (Ciechanover *et al.*, 1984; Finley *et al.*, 1987; Seufert and Jentsch, 1990), the unimpaired ability of the *ubr1-Δ1* mutant to degrade canavanine-containing proteins implies that the N-end rule-mediated recognition is only one of several targeting mechanism that mediate ubiquitin-dependent degradation of short-lived proteins. Indeed, a recent *in vitro* study has identified a ubiquitin-dependent pathway that degrades substrates with acetylated amino termini (Mayer *et al.*, 1989), which are not expected to be recognized by the N-end rule pathway.

Thus, the degradation of physiological substrates of the N-end rule pathway is either non-essential for cell growth and division or, alternatively, is essential, but can take place in the absence of the N-end rule pathway because the short-lived proteins involved each carry more than one degradation signal. This would account for the apparent absence of naturally short-lived yeast proteins that are metabolically stabilized in the *ubr1-Δ1* background. A precedent for this possibility is provided by the recent analysis of the naturally short-lived yeast MATα2 repressor that has been shown to possess two degradation signals operating via distinct, N-end rule-independent pathways (Hochstrasser and Varshavsky, 1990). If natural substrates of the N-end rule pathway do in fact carry more than one degradation signal, additional mutations (in the *ubr1-Δ1* genetic background) will be required to generate a conspicuous phenotype. We are testing this possibility by screening for 'synthetic' mutations (Basson *et al.*, 1987) i.e. those that are either lethal or otherwise phenotypically significant only in the absence of the functional *UBR1* gene (I.Ota, B.Bartel and A. Varshavsky, unpublished results).

One potential function for the N-end rule pathway that is compatible with the possiblities considered above is selective destruction of otherwise long-lived proteins that mislocalize into the cytosol from compartments such as the endoplasmic reticulum, Golgi, and vacuoles or lysosomes. It has been noted (Bachmair *et al.*, 1986) that the amino-terminal residues of compartmentalized proteins are largely of the destabilizing type according to the N-end rule, in striking

contrast to the amino-terminal residues of non-compartmentalized proteins, which are almost exclusively of the stabilizing type. The above conjecture is consistent with the proposed role of ubiquitin-dependent degradation in the maturation of erythrocytes, during which the destruction of previously compartmentalized proteins accounts for a large proportion of the total protein degradation (Dubiel and Rapoport, 1989). Metabolic instability of a small proportion of an abundant and long-lived compartmentalized protein would be difficult to detect using pulse–chase protocols and two-dimensional electrophoretic analyses. It is likely that the generation and analysis of synthetic mutants described above will provide evidence bearing on this hypothesis.

Materials and methods

Strains, media, genetic techniques and βgal assay

The *S.cerevisiae* strains used in this work are listed in Table I. *E.coli* strains MC1061 and JM101 (progagated in Luria Broth, LB) were used as hosts for plasmids and phage M13 derivatives. Rich (YPD) and synthetic yeast media were prepared according to Sherman *et al.* (1986), with synthetic media containing 0.67% yeast nitrogen base without amino acid (Difco) and either 2% glucose (SD medium) or 2% galactose as a carbon source. Synthetic medium plates supplemented with 0.1 M $KH_2PO_4-K_2HPO_4$ (pH 7.0) and 40 μg/ml 5-bromo-4-chloro-3-indole-β-D-galactoside (X-Gal) (Rose *et al.*, 1981) were used to assay yeast colonies for their relative βgal content. To induce the galactose-dependent expression of Ub-X-βgal proteins in the Gal$^-$ strains BBY45–BBY48 (see Table I, and Figures 3 and 7), Gal + Gro medium (Guarente *et al.*, 1982) containing 2% galactose, 2% ethanol, 2% glycerol and 40 μg/ml aspartic acid, along with 0.67% yeast nitrogen base without amino acids (Difco) and auxotrophic nutrients at concentrations recommended by Sherman *et al.* (1986) was used. Synthetic media lacking appropriate nutrient(s) were used to select for and maintain specific plasmids. Yeast mating, sporulation and tetrad analysis were performed as described by Sherman *et al.* (1986). Transformation of yeast was carried out by the lithium acetate method (Ito *et al.*, 1983). Strains were cured of *URA3* expressing plasmids using 5-fluoroorotic acid (5-FOA) as described by Boeke *et al.* (1984). Enzymatic activity of βgal in extracts was measured using *o*-nitrophenyl-β-D-galatoside (ONPG) as described by Ausubel *et al.* (1989).

Isolation of a ubr1 mutant

BWG9a-1 (Table I) was transformed with a 2μ-based, *URA3*-containing plasmid expressing Ub-Arg-βgal under control of the galactose inducible *GAL1* promoter (Bachmair *et al.*, 1986). These cells were mutagenized with EMS (Sherman *et al.*, 1986) to 4–8% survival and plated on synthetic medium containing 2% galactose and lacking uracil. After 3–4 days of growth at 23°C, colonies were replica plated onto X-Gal containing plates and allowed to grow at 23°C for 2 days. Since Arg-βgal is short-lived in wild-type cells (Figure 3B) its galactose-induced steady state level is low, resulting in white colonies on X-Gal plates. About 600 blue colonies (out of ~3 × 10^4 colonies screened) were picked and retested by a second round of replica plating. Extracts from 39 colonies that remained blue on the second X-Gal plate were tested for βgal activity using the ONPG assay (Ausubel *et al.*, 1989). Five apparent mutants that had significantly higher than wild-type levels of βgal activity were cured of the Ub-Arg-βgal plasmid using 5-FOA (Boeke *et al.*, 1984) and then transformed with plasmids expressing either Ub-Met-βgal, Ub-Pro-βgal or Ub-Arg-βgal (Bachmair *et al.*, 1986). A mutant (IW100), showing wild-type levels of βgal activity with Met-βgal and Ub-Pro-βgal but increased levels of βgal activity with Arg-βgal was chosen for further analysis. This strain was crossed to BWG1-7a; the resultant diploids had wild-type (low) levels of βgal activity, indicating that the relevant mutation in IW100 was recessive. Sporulation and tetrad analysis of the IW100/BWG1-7a diploid showed 2:2 segregation of high levels of Arg-βgal, indicating that a single lesion was responsible for the mutant phenotype. IW100 was back-crossed to wild-type strains (BWG1-7a or BWG9a-1) four times, to yield the strain IW104 carrying the original mutation. This strain was cured of the Ub-Arg-βgal plasmid and transformed with each of the 12 Ub-X-βgal plasmids (Bachmair and Varshavsky, 1989) that produce metabolically unstable X-βgals (X = Arg, Lys, His, Tyr, Phe, Leu, Ile, Trp, Glu, Gln, Asp, Asn). All of these normally short-lived X-βgals accumulated to high levels in the IW104 strain, in contrast to the slowly deubiquitinated Ub-Pro-βgal, which remained short-lived. Pulse–chase analysis followed by immunoprecipitation and electrophoretic fractionation of Arg-βgal (Bachmair *et al.*, 1986; see also Figure 3) confirmed that Arg-βgal was metabolically stabilized in IW104 (data not shown), whose relevant mutant gene was named *ubr1*.

Cloning the UBR1 gene

The *ubr1* strain IW104 carrying a Ub-Arg-βgal plasmid in which the *URA3* marker (Bachmair *et al.*, 1986) had been replaced by *LEU2* was transformed with a *S.cerevisiae* genomic DNA library carried in the *URA3*, *CEN4*-based vector YCp50 (Rose *et al.*, 1987). Approximately 2 × 10^3 transformants were screened for white colonies (low levels of Arg-βgal) on X-Gal plates that contained galactose and lacked uracil and leucine to maintain both plasmids. Four of the 10 initially selected white colonies remained white upon retesting on X-Gal plates. When these cells were cured of their library-derived plasmids on 5-FOA plates and retested, two of the four isolates regained their blue color (high levels of Arg-βgal) on X-Gal plates. Plasmid DNA isolated according to Hoffman and Winston (1987) from these two yeast strains was used to transform *E.coli* to ampicillin (amp) resistance. The YCp50 library-derived plasmids were distinguished from the Ub-Arg-βgal plasmid also present in the above strains by picking white *E.coli* transformants on LB + amp plates containing X-Gal. Indistinguishable library-derived plasmids (producing identically sized *Eco*RI and *Hin*dIII fragments) were obtained from both strains. This plasmid (pUBR1) was used to transform a *ubr1* (IW104) yeast strain, and it again complemented the *ubr1* mutatuon on X-Gal plates. Several subclones of pUBR1, including one containing the ~4 kb *Nsi*I fragment, one containing DNA from the *Bgl*II site to the 3′ end of the cloned insert, and one containing DNA from the *Xba*I site to the 5′ end of the cloned insert (Figure 2A), failed to complement the *ubr1* mutation.

To prove that the cloned insert originated from the chromosomal locus of the original *ubr1* mutation, we subcloned the ~2.5 kb *Hin*dIII fragment from pUBR1 that contains a unique *Xba*I site (Figure 2A) into *Hin*dIII-cut YIp33 (a yeast integrating plasmid containing the *LEU2* marker; Botstein *et al.*, 1979), to yield the plasmid pSOB6. This DNA was linearized with *Xba*I and used to transform the strain IW104 (Table I) carrying the Ub-Arg-βgal-expressing plasmid. Leu$^+$ transformants were selected, and the expected insertion was confirmed by Southern hybridization analysis. The transformants remained Ubr1$^-$ (i.e. they had high steady-state levels of Arg-βgal as judged by the colonies' color on X-gal plates) indicating that the lesion in the original *ubr1* allele did not map within the ~2.5 kb *Hin*dIII fragment which encompasses the 3′ portion of the *UBR1* reading frame (Figure 2A). Several transformants were crossed to a *MATα his4 ade1 ura3 leu2 UBR1* strain and the resultant diploids were sporulated and tetrads dissected. Eight tetrads in which all four segregants were Ura$^+$ (indicating that the Ub-Arg-βgal plasmid was maintained) were assayed for their Leu and Ubr phenotypes. All eight were of the parental ditype class (segregating 2 Leu$^-$ Ubr$^+$:2 Leu$^+$ Ubr$^-$), indicating that the integrated fragment from the cloned *ubr1* complementing DNA was closely linked to the *ubr1* locus.

DNA sequencing and Southern hybridization

Fragments (2–4 kb) of the 8.65 kb insert of the pUBR1 plasmid (Figure 2A) were subcloned into M13mp18 and M13mp19 (Ausubel *et al.*, 1989), and sets of nested deletions were generated using exoIII and exoVII (Özkaynak and Putney, 1987). Single-stranded M13 DNA was sequenced using the Sequenase kit (United States Biochemical Corp.) under conditions recommended by the manufacturer. The entire cloned insert in pUBR1 (Figure 2A) was sequenced on at least one strand, and the subset of this sequence shown in Figure 1 was sequenced on both strands. The predicted amino acid sequence of UBR1 was compared with sequences in the NBRF protein database (release 21.0) using the FastA algorithm, and to sequences (translated in all six reading frames) in the nucleic acid databases GenBank (release 60.0) and EMBL (release 19.0), using the TFastA algorithm.

For Southern hybridization analysis, DNA was isolated from yeast as described by Hoffman and Winston (1987), digested with restriction endonucleases, electrophoresed in 1% agarose gels in TAE buffer (Ausubel *et al.*, 1989), transferred to GeneScreen filters (New England Nuclear), and crosslinked to the filters using UV light (Church and Gilbert, 1984). DNA probes were labeled using [^{32}P]dCTP and the method of Feinberg and Vogelstein (1986). Hybridization was carried out at 42°C in 5 × SSPE (Sambrook *et al.*, 1989), 7% SDS and 40% formamide. Filters were washed as described for nylon membranes by Ausubel *et al.* (1989).

Plasmids and DNA manipulations

Plasmids were constructed by standard procedures (Ausubel *et al.*, 1989). DNA fragments were isolated from agarose gels using GeneClean (Bio101, La Jolla, CA). pUBR1 is the initial plasmid from the YCp50 based library (Rose *et al.*, 1987) that encodes the *UBR1* gene, with its 5′ end proximal

to the *Sph*I site of YCp50. Ub-X-βgal vectors were described previously (Bachmair *et al.*, 1986; Bachmair and Varshavsky, 1989).

To construct the *ubr1-Δ1* allele, the 1.2 kb *Sph*I−*Eco*RV fragment of pUBR1 (from the *Sph*I site in the YCp50 vector to the *Eco*RV site at position +149 in the UBR1-encoding insert) was ligated into *Sph*I/*Hin*cII-cut pUC19 (Ausubel *et al.*, 1989) to yield pSOB27. pSOB28 was made by filling in the ends of the ~2.9 kb *Cla*I fragment of pUBR1 (from the *Cla*I site at position +5087 in the UBR1-encoding insert to the *Cla*I site in the YCp50 vector), ligating it into *Sma*I-cut pUC19, and screening for constructs in which the *UBR1* coding region was proximal to the *Xba*I site of pUC19. The ~2.9 kb *Bam*HI−*Kpn*I fragment of pSOB28 was then ligated into *Bam*HI/*Kpn*I-cut pSOB27 to yield pSOB29, in which the insert of pUBR1 was modified by the ~5 kb deletion of a fragment from the 5′ *Eco*RV site to the 3′ *Cla*I site. The ends of the ~2.2 kb *Sal*I−*Xho*I fragment of YEp13 (Broach *et al.*, 1979) that contains the *LEU2* gene were filled in with Klenow Pol I, *Xba*I linkers were added, and the resultant fragment was ligated into the *Xba*I site of pSOB29, yielding pSOB30, which encodes *ubr1-Δ1*, the null *ubr1* allele (Figure 2A). The ~5 kb *Hin*dII fragment of pSOB30 that contained *ubr1-Δ1* was used to replace the wild-type *UBR1* allele in *S.cerevisiae* by homologous recombination (Rothstein, 1983) (see main text and the legend to Figure 2B).

Two plasmids overexpressing UBR1 were constructed. pSOB33 contained the ~8 kb *Sph*I−*Pst*I fragment of pUBR1 (from the *Sph*I site in the YCp50 vector to the *Pst*I site 3′ of the *UBR1* coding region) inserted into *Pst*I/*Sph*I-cut YEp352 (a 2μ based vector containing *URA3*; Hill *et al.*, 1986). pSOB35 was made by inserting the ~8 kb *Sal*I−*Pst*I fragment of pUBR1 (from the *Sal*I site in the YCp50 vector to the *Pst*I site 3′ of the *UBR1* coding region) into the *Sal*I/*Pst*I-cut YEplac112 (a 2μ-based vector containing *TRP3*; Gietz and Sugino, 1988).

To construct *UBR1−ha*, which encodes an epitope-tagged UBR1 (see main text), the ~2 kb *Xba*I−*Pst*I fragment encompassing the 3′ end of *UBR1* was ligated into the *Xba*I/*Pst*I-cut M13mp18 replicative form DNA (Ausubel *et al.*, 1989). A synthetic oligonucleotide (5′-GTAGAGGGCTTGAATCTAAGCGTAATCTGGAACATCGTATGGGTACCAAATCTCTCGCTCATC-3′), corresponding to the desired non-coding DNA strand, was used for insertional mutagenesis using the MutaGene kit (BioRad), yielding an open reading frame which encoded the carboxyl-terminal region of UBR1 followed by a nine-residue sequence, YPYDVPDYA, derived from hemagglutinin (ha) of the influenza virus, with a *Kpn*I site at the junction between the two coding regions. The ~2 kb *Xba*I−*Sph*I fragment of the resultant construct was ligated, along with the ~6 kb *Sal*I−*Xba*I fragment of pUBR1, into *Sal*I/*Sph*I-cut YEplac112, yielding the plasmid pSOB37, which expressed the UBR1−ha protein from the *UBR1* promoter.

Phenotypic characterization of the ubr1-Δ1 mutant

Assays measuring sensitivity of yeast cells to chronic heat stress (at 38 and 39°C), sensitivity to canavanine, and survival during stationary phase, nitrogen starvation and carbon starvation were carried out according to Finley *et al.* (1987). Sensitivity to acute heat stress was determined by exposing cells (growing exponentially in YPD at 30°C) to YPD at 50°C for 0−15 min prior to plating on YPD plates to assay colony formation at 30°C. Carbon sources tested included ethanol, acetate and glycerol. Ability to grow anaerobically was assayed on YPD plates incubated in GasPak pouches (Becton Dickinson) at 30°C. Freeze−thaw sensitivity was determined by freezing exponentially growing cells quickly (in liquid nitrogen) or slowly (in an isopropanol bath placed in a −85°C freezer), thawing the cells in a 30°C water bath, and determining plating efficiency on YPD at 30°C. Sensitivity to a cytotoxic concentration (15%) of ethanol was determined as described by Petko and Lindquist (1986) except that cells were grown in YPD prior to exposure. Bulk protein degradation in the presence and absence of canavanine was measured as described by Seufert and Jentsch (1990).

Pulse−chase and immunoprecipitation assays

Pulse−chase analysis, extraction and immunoprecipitation of βgal-based proteins with a monoclonal antibody to βgal were carried out essentially as described by Bachmair *et al.* (1986), except that cells were harvested by centrifugation at ~2000 *g* for 5 min, rather than by filtration. Briefly, yeast cells exponentially growing in Gal + Gro were labelled for 5 min at 30°C with ^{35}S-Translabel (ICN) and were then disrupted (either immediately or following a chase in the presence of 0.5 mg/ml cycloheximide and 10 mM cold methionine) by vortexing for 3 min at 4°C with 0.5 mm glass beads in 0.8 ml of cold buffer A (1% Triton X-100, 0.15 M NaCl, 5 mM Na-EDTA, 50 mM Na-HEPES pH 7.5) containing protease inhibitors (leupeptin, pepstatin A, antipain, aprotinin and chymostatin, each at 20 μg/ml, plus 1 mM phenylmethylsulfonyl fluoride and 5 mM *N*-ethylmaleimide). The extracts were centrifuged at 12 000 *g* for 5 min and portions of supernatants containing equal amounts of acid-insoluble ^{35}S were incubated for 1 h at 0°C with a monoclonal antibody to βgal (Bachmair *et al.*, 1986). 15 μl of protein A−Sepharose (Repligen) was added and the suspensions were incubated with rocking for 30 min at 4°C, followed by a 3 s centrifugation in a microcentrifuge. Pellets were washed three times with 0.8 ml of buffer A containing 0.1% SDS, resuspended in electrophoretic sample buffer, heated at 100°C for 3 min, and electrophoresed in a 6% polyacrylamide−SDS gel, followed by fluorography.

[^{35}S]methionine-labeled UBR1−ha was immunoprecipitated (Figure 6A) from yeast extracts prepared from BBY47 (*ubr1-Δ1*, Table I) transformed with either pSOB35 (*UBR1*) or pSOB37 (*UBR1−ha*). Ascitic fluid containing the anti-ha monoclonal antibody 12CA5 (Field *et al.*, 1988) was used, with the immunoprecipitation procedure described above for βgal, except that the antigen−antibody−protein A−Sepharose pellets were washed twice with 0.8 ml of 1% Triton X-100, 1 M NaCl, 5 mM Na-EDTA, 50 mM Na-HEPES pH 7.5 prior to a wash with 0.8 ml of Buffer A. Approximately 20-fold more UBR1−ha was immunoprecipitated from cells expressing it from a high copy (2μ-based) plasmid than from a low copy (*CEN*-based) plasmid under otherwise identical conditions (data not shown). The 12CA5 antibody-containing ascitic fluid was kindly provided by Ian Wilson and Gail Fieser, Scripps Clinic and Research Foundation.

For co-immunoprecipitation of UBR1−ha and a substrate protein (Figure 6B), the BBY47 (*ubr1-Δ1*) strain, either untransformed, transformed with pSOB35 (*UBR1*), or transformed with pSOB37 (*UBR1−ha*) was grown to A_{600} of ~2 in SD (lacking tryptophan in the case of plasmid bearing cells). Cells were pelleted by centrifugation at ~2000 *g* for 5 min, washed once with cold H_2O, resuspended in 1.5 vol of cold buffer B (0.1 M NaCl, 1 mM Na-EDTA, 50 mM Na-HEPES pH 7.5) with leupeptin, pepstatin A, antipain, aprotinin and chymostatin, each at 20 μg/ml. Cells were lysed by vortexing with an equal volume of glass beads for 4 min at 4°C. The extracts were centrifuged at 12 000 *g* for 10 min at 4°C, and the supernatants used as sources of either the ubiquitin-specific processing protease (BBY47), UBR1 protein (BBY47 + pSOB35), or UBR1−ha protein (BBY47 + pSOB37). ^{35}S-labeled Ub-Arg-βgal and Ub-Val-βgal proteins (~10^5 c.p.m./μg), purified as described by Gonda *et al.* (1989) from overexpressing *E.coli* (which does not deubiquitinate ubiquitin fusions), were kindly supplied by Erica Johnson (MIT). These ubiquitin fusions were deubiquitinated for 30 min at 30°C in 0.3 ml of buffer B containing ~2 × 10^6 c.p.m. of Ub-X-βgal and 30 μl of the extract prepared as described above from BBY47 (*ubr1-Δ1*). 70 μl of the resultant ^{35}S-labeled Arg- or Val-βgal proteins (~4.6 × 10^5 c.p.m.) was incubated for 1 h at 0°C with 30 μl of an extract (prepared as described above) containing either UBR1 or UBR1−ha along with a molar excess of the 12CA5 anti-ha monoclonal antibody, in a final volume of 0.2 ml of buffer B. 20 μl of protein A−Sepharose (Repligen) was added, and the suspensions were incubated with rocking for 30 min at 4°C, followed by a 3 s centrifugation in a microcentrifuge. Pellets were washed three times with 0.8 ml of buffer B containing 0.2% Triton X-100, resuspended in electrophoretic sample buffer, heated at 100°C for 3 min, and electrophoresed in a 6% polyacrylamide−SDS gel, followed by fluorography.

Protein crosslinking

Ub-Leu-DHFR and Ub-Met-DHFR were overexpressed in *E.coli*, metabolically labeled with [^{35}S]methionine, and purified using methotrexate affinity chromatography (Bachmair and Varshavsky, 1989; E.Johnson and A.Varshavsky, unpublished data). These ubiquitin fusions were deubiquitinated in a final volume of 0.45 ml of buffer B by incubating 0.15 ml (~2 × 10^6 c.p.m.) of Ub-X-DHFR for 1 h at 30°C with 30 μl of an extract prepared as described above from the BBY47 (*ubr1-Δ1*) yeast strain. 1.5 ml of buffer C (1 mM Na-EDTA, 25 mM Na-HEPES, pH 7.5) was added, and the protein was concentrated to a final volume of ~0.22 ml using Centricon-10 microconcentrators (Amicon). These extracts were used as a source of [^{35}S]X-DHFR in the crosslinking experiment below.

Extracts were prepared as described above from 0.5 l cultures (grown to A_{600} of ~2 in SD) for BBY47 (*ubr1-Δ1*) and BBY45 (*UBR1*) transformed with pSOB33 (a high copy plasmid expressing the UBR1 protein), and fractionated by precipitation with 30% saturated ammonium sulfate. The pellets (enriched, in the case of BBY45 + pSOB33, for the UBR1 protein) were dissolved in 1.5 ml of buffer C, followed by a 2-fold concentration using Centricon-30 microconcentrators (Amicon). For crosslinking (Hershko *et al.*, 1986), 20 μl of these unlabeled extracts was mixed with 50 μl (~4.5 × 10^5 c.p.m.) of [^{35}S]Leu-DHFR or [^{35}S]Met-DHFR (see above), with or without the addition of crosslinker [7 μl of 10 mM *bis* (sulfosuccinimidyl) suberate (Pierce)]. The samples were incubated for 15 min at 0°C. Crosslinking was stopped with 10 mM ethanolamine pH 8.0, followed by the addition of electrophoretic sample buffer, heating at 100°C for 3 min, electrophoresis in a 5% polyacrylamide−SDS gel, and fluorography.

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References

Arfin,S.M. and Bradshaw,R.A. (1988) *Biochemistry*, **27**, 7979–7984.
Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds), (1989) *Current Protocols in Molecular Biology*. John Wiley and Sons, New York.
Bachmair,A. and Varshavsky,A. (1989) *Cell*, **56**, 1019–1032.
Bachmair,A., Finley,D. and Varshavsky,A. (1986) *Science*, **234**, 179–186.
Banuett,F., Hoyt,M.A., McFarlane,L., Echols,H. and Herskowitz,I. (1986) *J. Mol. Biol.*, **187**, 213–224.
Basson,M.E., Moore,R.L. O'Rear,J. and Rine,J. (1987) *Genetics*, **117**, 645–655.
Boeke,J.D., LaCroute,F. and Fink,G.R. (1984) *Mol. Gen. Genet.*, **197**, 345–346.
Botstein,D., Falco,S.C., Stewart,S.E., Brennan,M., Scherer,S., Stinchcomb,D.T., Struhl,K. and Davis,R.W. (1979) *Gene*, **8**, 17–24.
Broach,J.R., Strathern,J.N., and Hicks,J.B. (1979) *Gene*, **8**, 121–133.
Chau,V., Tobais,J.W., Bachmair,A., Marriott,D., Ecker,D.J., Gonda,D.K. and Varshavsky,A. (1989) *Science*, **243**, 1576–1583.
Chiang,H.-L., Terlecky,S.R., Plant,C.P. and Dice,J.F. (1989) *Science*, **246**, 382–385.
Church,G.M. and Gilbert,W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1991–1995.
Ciechanover,A. and Schwartz,A.L. (1989) *Trends Biochem. Sci.*, **14**, 483–488.
Ciechanover,A., Finley,D. and Varshavsky,A. (1984) *Cell*, **37**, 57–66.
Dubiel,W. and Rapoport,S.M. (1989) In Knecht,E. and Grisolia,S. (eds), *Cell Biology Review*. Springer International, Volume 21, pp. 505–521.
Evans,T., Rosenthal,E.T., Youngblom,J., Distel,D. and Hunt,T. (1983) *Cell*, **33**, 389–396.
Feinberg,A.P. and Vogelstein,B. (1984) *Anal. Biochem.*, **137**, 266–267.
Field,J., Nikawa,J.-I., Broek,D., MacDonald,B., Rodgers,L., Wilson,I.A., Lerner,R.A. and Wigler,M. (1988) *Mol. Cell. Biol*, **8**, 2159–2165.
Finley,D., Özkaynak,E. and Varshavsky,A. (1987) *Cell*, **48**, 1035–1046.
Finley,D., Özkaynak,E., Jentsch,S., McGrath,J.P., Bartel,B., Pazin,M., Snapka,R.M. and Varshavsky,A. (1988) In Rechsteiner,M. (ed.), *Ubiquitin*. Plenum Press, New York, pp. 39–75.
Finley,D., Bartel,B. and Varshavsky,A. (1989) *Nature*, **338**, 394–401.
Frankel,A.D., Chen,L. Cotter,R.J. and Pabo,C.O. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6297–6300.
Geitz,R.D. and Sugino,A. (1988) *Gene*, **74**, 527–534.
Gonda,D.K., Bachmair,A., Wünning,I., Tobias,J.W., Lane,W.S. and Varshavsky,A. (1989) *J. Biol. Chem.*, **264**, 16700–16712.
Guarente,L., Yocum,R.R. and Gifford,P. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7419–7414.
Hershko,A. (1988) *J. Biol. Chem.*, **263**, 15237–15240.
Hershko,A., Heller,H., Elias,S. and Ciechanover,A. (1983) *J. Biol. Chem.*, **258**, 8206–8214.
Hershko,A., Heller,H., Eytan,E. and Reiss,Y. (1986) *J. Biol. Chem.*, **261**, 11992–11999.
Hill,J.E., Myers,A.M., Koerner,T.J. and Tzagoloff,A. (1986) *Yeast*, **2**, 163–167.
Hochstrasser,M. and Varshavsky,A. (1990) *Cell*, **61**, 697–708.
Hoffman,C.S. and Winston,F. (1987) *Gene*, **57**, 267–272.
Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol*, **153**, 163–168.
Jentsch,S., Seufert,W., Sommer,T. and Reins,H.-A. (1990) *Trends Biochem. Sci.*, **15**, 195–198.
Kulesh,D.A., Cecena,G., Darmon,Y.M., Vasseur,M. and Oshima,R.G. (1989) *Mol. Cell. Biol.*, **9**, 1553–1565.
Landschulz,W.H., Johnson,P.F. and McKnight,S.L. (1988) *Science*, **240**, 1759–1764.
Maicas,E. Pluthero,F.G. and Friesen,J.D. (1988) *Mol. Cell Biol.*, **8**, 169–175.
Mayer,A., Siegel,N.R., Schwartz,A.L. and Ciechanover,A. (1989) *Science*, **244**, 1480–1483.
Munro,S. and Pelham,H.R.B. (1984) *EMBO J.*, **3**, 3087–3093.
Murray,A.W., Solomon,M.J. and Kirschner,M.W. (1989) *Nature*, **339**, 280–286.
Olson,T.S. and Dice,J.F. (1989) *Curr. Opinion Cell Biol.*, **1**, 1194–1200.
Özkaynak,E. and Putney,S.D. (1987) *Biotechniques*, **5**, 770–773.
Petko,L. and Lindquist,S. (1986) *Cell*, **45**, 885–894.
Pickart,C.M. and Rose,I.A. (1985) *J. Biol. Chem.*, **260**, 1573–1581.
Reiss,Y. and Hershko,A. (1990) *J. Biol. Chem.*, **265**, 3685–3690.
Reiss,Y., Kaim,D. and Hershko,A. (1988) *J. Biol. Chem.*, **263**, 2693–2698.
Reiss,Y., Heller,H. and Hershko,A. (1989) *J. Biol. Chem.*, **264**, 10378–10383.
Rose,M., Casadaban,M.J., and Botstein,D. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2460–2464.
Rose,M.D., Novick,P., Thomas,J.H., Botstein,D. and Fink,G.R. (1987) *Gene*, **60**, 237–243.
Rothstein,R. (1983) *Methods Enzymol.*, **101**, 202–211.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Seufert,W. and Jentsch,S. (1990) *EMBO J.*, **9**, 543–550.
Sharp,P.M. and Li,W.-H. (1987) *Nucleic Acids Res.*, **15**, 1281–1295.
Sherman,F., Fink,G.R. and Hicks,J.B. (1986) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Straus,D.B., Walter,W.A. and Gross,C.A. (1987) *Nature*, **329**, 348–351.
Townsend,A., Bastin,J., Gould,K., Brownlee,G., Andrew,M., Coupar,B. Boyle,D., Chan,S. and Smith,G. (1988) *J. Exp. Med.*, **168**, 1211–1224.
Tsay,Y.-F., Thompson,J.R., Rotenberg,M.O., Larkin,J.C. and Woolford,Jr,J.L. (1988) *Genes Dev.*, **2**, 664–676.
Varshavsky,A., Bachmair,A., Finley,D., Gonda,D. and Wünning,I. (1988) In Rechsteiner,M. (ed.), *Ubiquitin*. Plenum Press, New York, pp. 287–321.

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The UBR1 sequence will appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number X53747.